Nitroxide Tempo, a Small Molecule, Induces Apoptosis in Prostate Carcinoma Cells and Suppresses Tumor Growth in Athymic Mice

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BACKGROUND. In previous studies, nitroxide tempo (2, 2, 6, 6-tetramethyl-piperidine-1-oxyl), a small molecule, induced cell death in cancer cells. The current study examined the antineoplastic properties of tempo in the human hormone-dependent/hormone-independent prostate carcinoma models (LNCaP, DU-145, and PC-3).

METHODS. The apoptotic effects of tempo were examined by the flow cytometric analysis of cells labeled with fluorescein isothiocyanate-conjugated annexin-V, and by electron microscopy. Enzymatic assays were performed to measure the activities of 2 cysteine proteases, i.e., caspase-9 and caspase-3, in tempo-treated cells. The effects of tempo on cell proliferation and on cell cycle distribution profiles were measured by the flow cytometric assay using immunofluorescent staining of incorporated 5'-bromo-2'-deoxyuridine (BrdU) coupled with 7-amino-actinomycin D (7-AAD) staining of total DNA. The number of proliferating cells was also determined independently by enzyme-linked immunosorbent assay using chemiluminescent detection of incorporated BrdU. Subcutaneous growth of human prostate carcinoma in athymic mice was monitored after intratumoral administration of tempo into tumor-bearing mice. In addition, cell viability assays were performed to compare the cytotoxic effect of a combination of doxorubicin or mitoxantrone and tempo with single agents.

RESULTS. Tempo treatment of prostate carcinoma cells caused a significant increase in the number of apoptotic cells compared with control groups (tempo, 2.5 mM, 24 hours: DU-145, approximately 3.4-fold; PC-3, approximately 6–7-fold; tempo 1 mM, 24 hours: LNCaP, approximately 12-fold). Tempo-induced loss of cell viability was blocked partially or completely after pretreatment of cells with actinomycin-D or cycloheximide, suggesting a de novo macromolecule synthesis-dependent mechanism of cell death. Electron microscopy revealed aggregation and marginalization of chromatin in the nuclei of a large number of tempo-treated LNCaP cells. Tempo treatment of LNCaP cells resulted in enhanced activities of caspase-9 (tempo, 5 mM, 15 hours: approximately 2-fold) and caspase-3 (tempo, 2.5 mM, 24 hours: approximately 12-fold). Tempo treatment also led to an enhanced number of cells in G_2/M phase of the cell cycle (tempo, 5.0 mM, 24 hours: DU-145, approximately 1.6-fold; PC-3, approximately 1.5-fold; LNCaP, approximately 5.3-fold), and decreased BrdU incorporation indicative of a decline in the number of proliferating cells (tempo, 2.5 mM, 24 or 48 hours; DU-145, approximately 2–3-fold; PC-3, approximately 1.2-fold; LNCaP, approximately 5–10-fold). Administration of tempo into LNCaP tumor-bearing mice resulted in a significant inhibition of tumor growth (percent initial tumor volume [Day 30, $n = 4$]: vehicle, 845.35 ± 272.83; tempo, 9.72 ± 9.72; tempo vs. vehicle, $P < 0.02$). In hormone-refractory prostate carcinoma cells, a combination of relatively low doses of tempo and doxorubicin or mitoxantrone caused enhanced cytotoxicity as compared with single agents.

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CONCLUSIONS. These data demonstrated that nitroxide tempo induced apoptosis and activated a caspase-mediated signaling pathway in prostate carcinoma cells. Tempo treatment also caused cell cycle arrest in G_2/M phase and decreased the number of proliferating cells (S phase). Tempo treatment of tumor-bearing mice led to inhibition of tumor growth, suggesting that tempo is a novel member of the small-molecule family of antineoplastic agents. *Cancer* 2005;103:1302–13.

KEYWORDS: nitroxide tempo, prostate carcinoma, apoptosis, caspases, cell cycle and cell proliferation, antitumor effect, in vitro chemosensitization.

Metastatic prostate carcinoma is associated with high morbidity and mortality with a median survival of approximately 12–15 months.[1] The metastatic lesions are composed of a heterogeneous population of androgen-sensitive and androgen-resistant tumor cells. Although androgen-ablation therapy activates apoptotic death of androgen-dependent cancer cells, it has no effect on hormone-independent cancer cells.[2,3] Chemotherapeutic agents (e.g., estramustine, vincristine, cytoxan, and doxorubicin) used singly, which primarily target cells with high rates of proliferation, have resulted in response rates of < 10% in patients with advanced prostate carcinoma.[4] Randomized studies using a combination of mitoxantrone, an anthracyclin, and corticosteroids have shown palliative responses with no improvements in survival. Failed responses to chemotherapy have been attributed, in part, to an increased survival capacity or resistance of androgen-independent cells to apoptosis.[2] Identification of small, membrane-permeable molecules capable of activating potent apoptotic signals in both hormone-sensitive and insensitive malignant cell populations offers the prospect of a curative therapy for patients with metastatic prostate carcinoma.

Nitroxide compounds are low molecular weight, membrane-permeable, nonimmunogenic, and electron paramagnetic resonance (EPR)-detectable and stable-free radicals.[5–8] In tissue specimens, nitroxides are reduced rapidly, primarily in the mitochondria through enzyme-dependent mechanisms, to hydroxylamines. The oxidation of hydroxylamines to nitroxides also can occur, resulting in the distribution of nitroxides/hydroxylamines that are dependent on oxygen status and reducing equivalents.[9–11] Nitroxides exert antioxidant and prooxidant effects.[6,7,12–15] These compounds are cleared quite rapidly from the circulation and from the whole body, and persistent conjugants have been synthesized to elucidate their long-term biologic effects.[16–18]

We previously reported that tempo (2, 2, 6, 6-tetramethyl-piperidine-1-oxyl), a low molecular weight nitroxide-free radical, induces apoptosis in breast carcinoma cells.[19] The mechanisms of tempo-induced apoptosis and antineoplastic activity are not known. In the current study, we examined the effects of tempo on cell survival, caspase activity, cell cycle, and cell proliferation in human prostate carcinoma cells. The antitumor effect of tempo was investigated in the athymic mice model of human prostate carcinoma. The cytotoxic effect of a combination of doxorubicin or mitoxantrone and tempo was compared with single agents in hormone-responsive and hormone-refractory prostate carcinoma cell lines. Our data provide evidence of tempo as a novel anticancer agent and demonstrate that combination with tempo improves the efficacy of certain chemotherapeutic drugs.

MATERIALS AND METHODS

Cell Cultures and Treatments

Human prostate carcinoma cells (PC-3 and DU-145) were grown in 75-cm^2 tissue flasks in improved minimum essential medium (Biofluids, Biosource International, Rockville, MD) containing 10% bovine calf serum (BCS) supplemented with 2 mM L-glutamine in a humidified atmosphere of 5% CO_2/95% air at 37 °C. LNCaP prostrate carcinoma cells were grown in RPMI 1640 medium (Biofluids) containing 10% BCS and 2 mM L-glutamine under the same conditions. Tempo, cycloheximide (CHX), and actinomycin-D (ACT-D; Aldrich-Sigma, St. Louis, MO) were reconstituted in 100% ethanol. Doxorubicin (HandeTech Development Co., Houston, TX) was reconstituted in 100% dimethylsufoxide. Mitoxantrone (NSC 301739, MSD 5001-07), kindly provided by Dr. Robert J. Schultz (Drug Synthesis and Chemistry Branch, National Cancer Institute [NCI], National Institutes of Health, Frederick, MD), was dissolved in normal saline. These compounds were diluted to desired concentrations in appropriate culture medium containing 5% BCS before treatment.

Apoptosis Assay

The ApoAlert annexin-V apoptosis detection system (Clontech, Palo Alto, CA) was used to measure the relative distribution of early apoptotic and late apoptotic/necrotic cells. Briefly, cells (1×10^6) were

grown overnight in medium containing 10% BCS in T-25 flasks (in triplicate per treatment point), and switched to medium containing 5% BCS ± tempo at various concentrations for the indicated times. After treatment, cells were washed 3 times with phosphate-buffered saline (PBS), trypsinized, and resuspended in 2 volumes of PBS, followed by centrifugation at 3000 × g for 5 minutes. The cell pellet was washed once with PBS and resuspended in 200 μL of 1 × binding buffer (Clontech). The cell suspension was double labeled with fluorescein isothiocyanate (FITC)-conjugated annexin-V and propidium iodide (PI) according to the manufacturer's instructions. Annexin-V and PI uptake were detected using the FACSORT flow cytometer (Becton Dickinson, San Jose, CA), and data were analyzed using FCS Express software (De Novo Software; Thornhill, OT, Canada). A minimum of 100,000 events were collected. FITC-negative and PI-negative cells were scored as viable cells, FITC-positive and PI-negative cells were scored as early apoptotic cells, and FITC-positive and PI-positive cells were scored as late apoptotic/necrotic cells. Untreated cells (unstained cells, annexin-V/FITC-labeled cells, PI-labeled cells, and annexin-V/FITC/PI-labeled cells) were used as background controls.

Cell Viability Assay

Cell viability was determined by a quantitative colorimetric assay using WST-1[2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, sodium salt] (Roche, Indianapolis, IN). The assay is based on cleavage of tetrazolium salt to formazan by metabolically active cells and increased absorbance at 450 nm correlates with the number of viable cells. Briefly, 2×10^4 cells were seeded per well in a 96-well flat-bottom plate. For combination treatment of tempo and CHX or ACT-D, cells were pretreated with the desired concentration of either ACT-D or CHX for the indicated time, followed by the addition of tempo to the same medium. For the tempo and anticancer drug combination study, cells were treated simultaneously with tempo ± doxorubicin or tempo ± mitoxantrone. At the selected time point after treatment, 20 μL of WST-1 reagent was added to each well containing 200 μL of medium according to the manufacturer's instructions. The plate was incubated for 1 hour at 37 °C and read at an absorbance of 450 nm and a reference wavelength of 650 nm by a Vmax microtiter plate reader (Molecular Devices, Sunnydale, CA).

Electron Microscopy

LNCaP cells (1×10^6) were seeded overnight onto 25-mm^2 tissue culture flasks in medium containing 10% BCS. Cells were treated with medium containing 5% BCS ± tempo (5 mM) for 24 hours. After tempo treatment, monolayer cells were washed three times with PBS and fixed in 2.5% glutaraldehyde/3% paraformaldehyde in PBS for 30 minutes. Postfixation was performed in 1% osmium tetroxide in distilled water for 1 hour, sequentially followed by washing 3 times in distilled water, dehydration in ethanol, treatment with propylene oxide, and embedding in epoxy resin as described earlier.[20] Ultrathin sections (1 μm) were stained with 2% uranyl acetate and lead acetate for 30 minutes in the dark and stained ultrathin sections were examined under a JEOL 1200 EX transmission electron microscope (JEOL USA; Peabody, MA). Untreated or ethanol (0.1%)-treated cultures were used as controls.

Caspase Activity Assays

LNCaP cells were treated with tempo and assayed for caspase-9 and caspase-3 activities using Ac-LEHD-AFC caspase 9-like substrate (Calbiochem, San Diego, CA) and ApoAlert CPP32/caspase-3 activity assay kit (Clontech). In brief, cells were cultured in 100-mm culture dishes containing RPMI medium supplemented with 10% BCS (approximately 3×10^6 per dish, in triplicate per treatment point). Cells were treated with the desired concentration of tempo in medium containing 5% BCS for various times, sequentially followed by washing 3 times with ice-cold PBS, lysis in cell lysis buffer (Clontech) for 10 minutes on ice, and microcentrifugation at 15,000 × g for 15 minutes. Whole cell extracts (50 μg of protein) were incubated for 1 hour at 37 °C in reaction mixture containing 10 mM dithiothreitol in 2 × reaction buffer and 50 μM of fluorogenic substrates: Ac-LEHD-AFC (caspase-9) or Ac-DEVD-AMC (caspase-3). Fluorescence was measured by a fluorimeter (Hitachi F4500; San Jose, CA) with excitation set at 400 nm and emission set at 505 nm. Fold change in caspase activity represented the fluorescence ratio of lysate treated with tempo to vehicle control lysate (0.1% ethanol).

Coupled Cell Cycle and Cell Proliferation Assay

A 5'-bromo-2'-deoxyuridine (BrdU) flow kit (BD Pharmingen, San Diego, CA) was used to determine the cell cycle kinetics and to measure the incorporation of BrdU into DNA of proliferating cells. The assay was performed according to the manufacturer's protocol. Briefly, cells (1×10^6 per well) were seeded overnight in 6-well tissue culture plates and treated with a desired concentration of tempo in medium containing 5% BCS for 20 hours or left untreated, followed by addition of 10 μM BrdU, and incubations continued for an additional 4 hours. Both floating and adherent cells were pooled from triplicate wells per

treatment point, fixed in a solution containing paraformaldehyde and the detergent saponin, and incubated for 1 hour with DNAase at 37 °C (30 μg per sample). FITC-conjugated anti-BrdU antibody (1:50 dilution in Wash buffer; BD Pharmingen, San Diego, CA) was added and incubation continued for 20 minutes at room temperature. Cells were washed in Wash buffer and total DNA was stained with 7-amino-actinomycin D (7-AAD; 20 μL per sample), followed by flow cytometric analysis using FACSort (Becton Dickinson). BrdU content (FITC) and total DNA content (7-AAD) were determined using Cell Quest (Becton Dickinson) and FCS Express software (De Novo Software).

Cell Proliferation Assay

A cell proliferation enzyme-linked immunosorbent assay, BrdU (chemiluminescence) kit (Roche Diagnostics GmbH, Mannheim, Germany) was used to measure the incorporation of BrdU during DNA synthesis according to the manufacturer's protocol. Briefly, cells were seeded overnight in black 96-well tissue culture plates with clear, flat bottoms (Becton Dickinson) at a density of 10,000 cells per well in 100 μL medium containing 10% BCS (4–6 wells per treatment point). Cells were treated with the desired concentrations of tempo or vehicle control (0.1% ethanol) for 24 or 48 hours in medium containing 5% BCS. BrdU (10 μM) was added to the culture medium 2 hours before the termination of tempo treatment. BrdU-labeled adherent cells were fixed and DNA was denatured in FixDenat (Roche Diagnostics) for 30 minutes at room temperature. Cells were incubated with peroxidase-conjugated anti-BrdU antibody (anti-BrdU-POD) for 90 minutes at room temperature and washed 3 times with PBS. The immune complex was detected by the luminol substrate reaction, followed by measurement of chemiluminescence using a luminometer (MicroLumat Plus, LB 96V with WinGlow software, Berthold Technologies, Bad Wildbad, Germany).

Antitumor Efficacy Protocol

The antitumor activity of tempo was tested in the LNCaP prostate tumor model. Animal studies were performed at the Animal Resource Research Facility (Division of Comparative Medicine, Georgetown University, Washington, DC) in compliance with the institutional guidelines for animal care and use.

For tumor cell inoculations, logarithmically growing LNCaP cells (1×10^6) were inoculated subcutaneously with 200 μL Matrigel (>10 mg/mL protein, Becton Dickinson Labware, Bedford, MA) in the right flank region of 4–6-week-old male BALB/c nu/nu mice (NCI). Treatments began when tumor volumes reached a mean of approximately 75–100 mm^3 as measured by a caliper and determined by the formula length × width × height divided by 2, an estimated value for the volume of an ellipse (π lwh/6). Tumor-bearing animals were randomized as treatment or control groups ($n = 4$).

Mice-bearing LNCaP tumor xenografts received tempo intratumorally, once per day, 100 mg/kg per dose, × 8 (Days 1–8), followed by 200 mg/kg per dose, × 15 (Days 9–23). Control groups received 4% ethanol in PBS or PBS at the same dosing schedule as tempo. Tumor sizes were monitored 2–3 times weekly and individual percent initial tumor volume was expressed as the percentage of pretreatment tumor volume at Day 0 (100%). Mean percent initial tumor volume ± standard error was plotted. The animals were humanely euthanized when the tumor volumes exceeded the recommended tumor burden guidelines.

Statistical Analysis

One-way analysis of variance was used to determine statistical difference between mean percent initial tumor volumes in various treatment and control groups. $P < 0.05$ was a significant value.

RESULTS

Tempo Induces Apoptosis in Prostate Carcinoma Cells

Androgen-independent (DU-145, PC-3) and androgen-dependent human prostate carcinoma cells (LNCaP) were treated with tempo as indicated in Figure 1. The cells were stained with two cell markers, annexin V to measure phosphatidyl-serine translocation to the extracellular leaflets (early apoptosis) and PI to measure the loss of phospholipid membrane integrity (late apoptosis/necrosis). Treatment of DU-145 cells with 2.5 mM tempo for 24 hours resulted in an increase in both early apoptotic cells and late apoptotic/necrotic cells (right panel) compared with controls (untreated, left panel; ethanol vehicle [0.1%], middle panel; Fig. 1A). The time-course of tempo-induced apoptosis in DU-145 cells is shown in Figure 1B. Treatment of PC-3 cells with tempo for 24 hours also resulted in a dose-dependent increase in apoptotic cell death (Fig. 1C). In LNCaP cells, apoptosis occurred early and with a greater magnitude with tempo at 5 mM (4 hours) compared with tempo at 1 mM (24 hours; Fig. 1D). The sensitivity of tempo in these tumor cells appears to be cell type dependent, with LNCaP cells exhibiting the greatest sensitivity (12-fold, 1 mM, 24 hours; 15-fold, 5 mM, 4 hours), followed by PC-3 cells (6.7-fold, 2.5 mM, 24 hours) and DU-145 cells (3.4-fold, 2.5 mM, 24 hours). These data suggest that the apoptotic effects induced by tempo are time and dose dependent, and reproducible in all androgen-dependent and in-

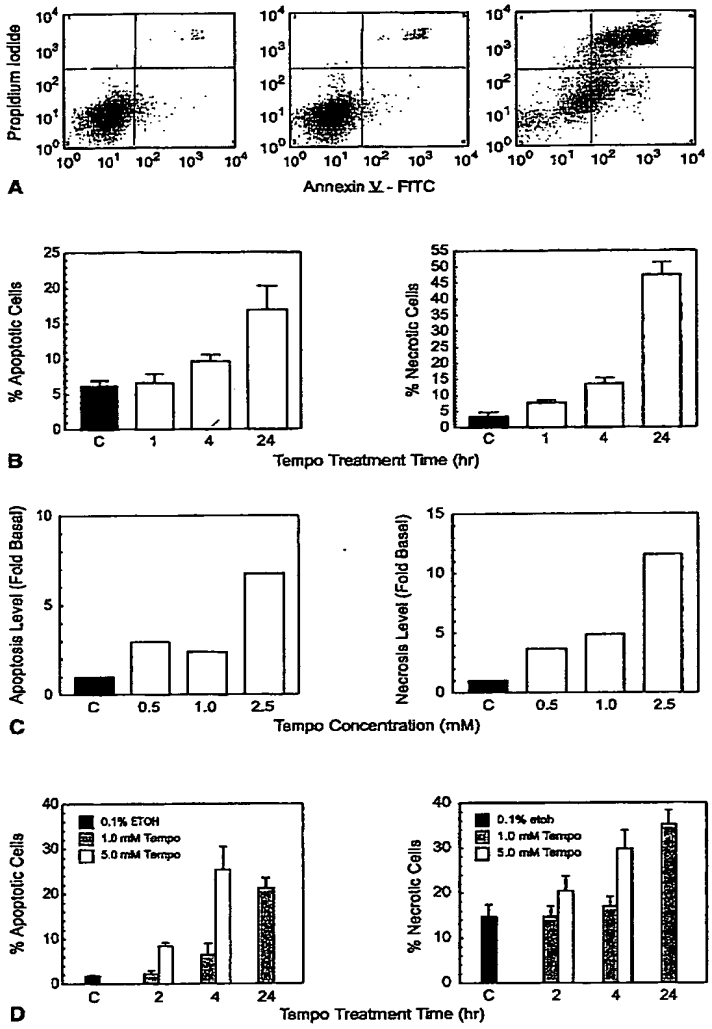

FIGURE 1. Time course and dose-response experiments showing tempo-induced apoptosis in prostate carcinoma cells, DU-145 (A and B), PC-3 (C), and LNCaP (D). Cells (1×10^6) were cultured in T-25 flasks overnight in medium containing 10% bovine calf serum (BCS) and switched to medium containing 5% BCS before tempo treatment as indicated. Cells were double stained with annexin V-fluorescein isothiocyanate (FITC) and propidium iodide and analyzed by flow cytometry as described in the Materials and Methods. (A) Representative cytograms of untreated (left panel), 0.1% ethanol-treated (24 hours, middle panel), and tempo-treated cells (2.5 mM, 24 hours) (right panel). Each panel shows the relative distribution of viable (lower left quadrant), early apoptotic cells (lower right quadrant), and late-stage apoptotic/necrotic cells (upper right quadrant). (B–D) Bar graphs showing percent early apoptosis (right panels) and percent late apoptosis/necrosis (left panels) in prostate carcinoma cells treated with tempo compared with control treatment (0.1% ethanol). DU-145 cells were treated with 2.5 mM tempo for the indicated times (B), PC-3 cells were treated with the indicated doses of tempo for 24 hours (C), and LNCaP cells were treated with the indicated doses of tempo for various times (D). Values shown are mean ± the standard deviation of triplicate determination in each category and data represent one of three independent experiments.

dependent human prostate carcinoma cell lines examined.

Pretreatment of Prostate Carcinoma Cells with Actinomycin-D or Cycloheximide Inhibits Cell Death Induced by Tempo

To investigate whether tempo-induced cell death requires de novo synthesis of RNA or protein, we pretreated LNCaP cells with either ACT-D (an RNA synthesis inhibitor) or CHX (a protein synthesis inhibitor) before challenging the cells with tempo. The protective effects of these antimetabolites are reported to be concentration and cell system dependent.[21] Concentrations of ACT-D, 10–50 ng/mL, and of CHX, 1–10 μg/mL, have been shown to be effective in blocking de novo macromolecule synthesis without compromising the viability of the cells.[22,23] A dose-dependent loss of cell viability was observed in prostate carcinoma cells treated with tempo (Fig. 2A). Pretreatment of LNCaP cells with ACT-D (10 ng/mL, 2 hours) before the addition of a cytotoxic dose of tempo (5 mM, 5 hours) completely restored the viability of LNCaP cells (0.1% ethanol control, 100%; ACT-D, 100.81 ± 0.74%; ACT-D and tempo, 97.35 ± 2.8%; tempo, 60.74 ± 10.52%; Fig. 2B). The protective effect in LNCaP cells occurred at ACT-D concentrations between 10 ng/mL and50 ng/mL (data no shown). In parallel experiments (Fig. 2C), a partial restoration of cell viability also was observed in LNCaP cells treated with CHX (0.5 μg/mL, 1 hour), followed by a cytotoxic dose of tempo (5 mM, 4 hours), CHX and tempo (72.41 ± 12.86%), and tempo (35.20 ± 7.16%). It is noteworthy that we found that the viability of PC-3 cells (Fig. 2C) was restored completely with CHX pretreatment (2.5 ng/mL, 4 hours),

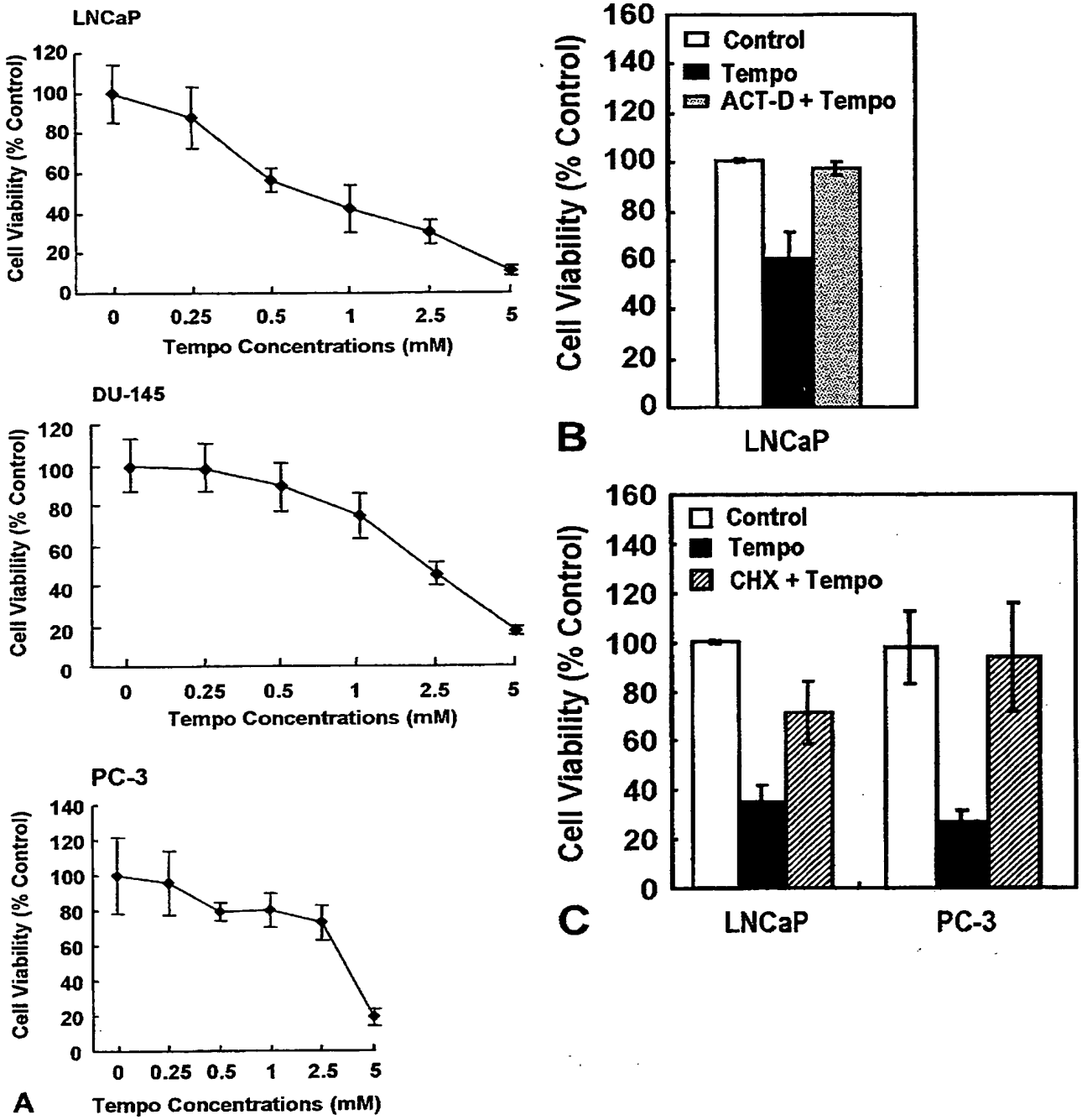

FIGURE 2. Pretreatment with actinomycin D (ACT-D) or cycloheximide (CHX) restores viability in prostate carcinoma cells treated with tempo. (A) Dose-dependent loss of cell viability in tempo-treated prostate carcinoma cells. Prostate carcinoma cells were treated with the indicated doses of tempo for 24 hours (LNCaP and PC-3) or 48 hours (DU-145). Control cells were treated with 0.1% ethanol for the duration of the experiment. Cell viability was determined by the WST-1 assay. Each data point represents the mean ± the standard deviation (SD) of four to six determinations. (B) LNCaP cells were pretreated with ACT-D (10 ng/mL, 2 hours) and incubations continued with or without tempo (5 mM) for an additional 5 hours. (C) LNCaP and PC-3 cells were pretreated with CHX (LNCaP, 0.5 μg/mL, 1 hour; PC-3, 2.5 ng/mL, 4 hours) and incubations continued in the absence or presence of tempo (LNCaP, 5 mM, 4 hours; PC-3, 0.225 mM, 16 hours). The cell viability was determined by WST-1 assay as described in the Materials and Methods. Control cells were treated with 0.1% ethanol. Values shown are mean ± SD of six determinations in each treatment category. Data represent one of three independent experiments.

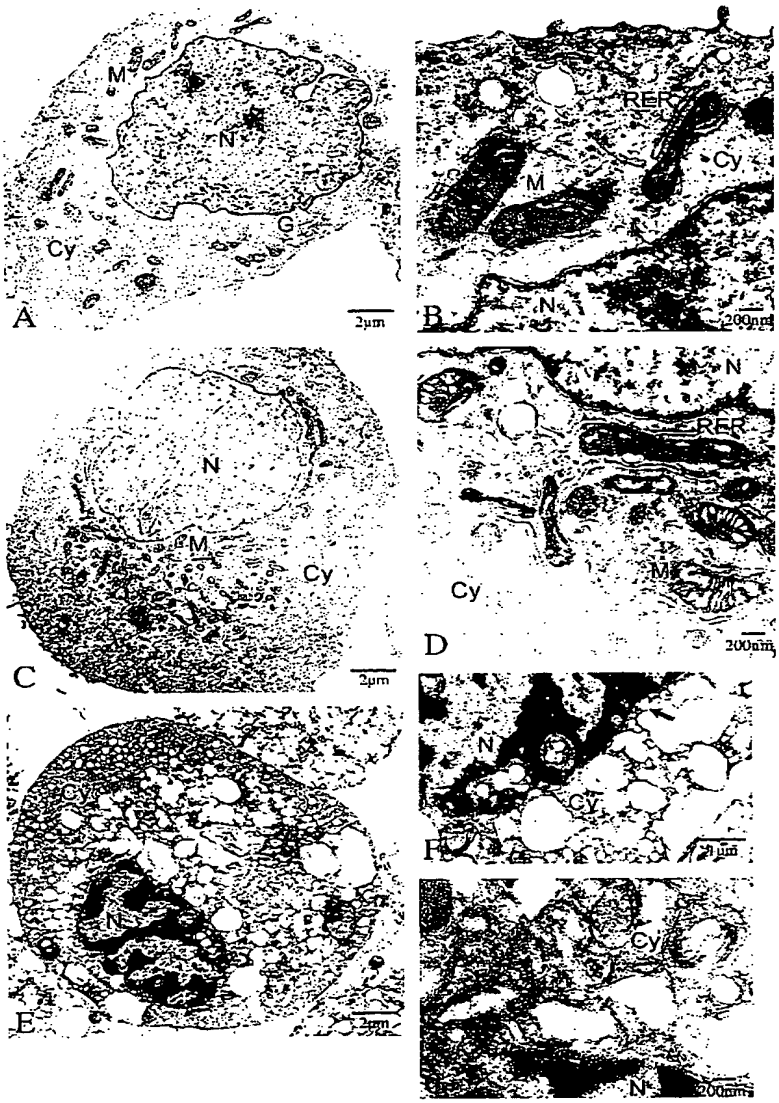

FIGURE 3. Tempo induces chromatin fragmentation in LNCaP cells. LNCaP cells were treated with tempo and transmission electron microscopy was performed as detailed in the Materials and Methods. (A) untreated cells. (B) A portion of the cell shown in Figure 3A. (C) 0.1% ethanol, 24 hours. (D) A portion of the cell shown in Figure 3C. (E) 5 mM tempo, 24 hours. (F) A portion of the cell shown in Figure 3E. (G) A portion of another cell treated with tempo (5 mM, 24 hours). M: mitochondria; G: Golgi apparatus; RER: rough endoplasmic reticulum; Cy: cytoplasm; N: nucleus. The arrow in F indicates swelling in the nuclear membrane. Original magnification × 4000 (A, C, E); × 8000 (F); × 20,000 (B, D, F).

followed by a cytotoxic dose of tempo (0.23 mM, 16 hours; 0.1% ethanol control, 100 ± 7.94%; CHX, 97.76 ± 14.84%; CHX and tempo, 93.61 ± 22.17%; and tempo, 26.62 ± 5.04%). Similar results were observed in human breast adenocarcinoma MDA-MB 231 cells challenged with tempo (data not shown). These data suggest that cell death induced by tempo may require de novo RNA and/ or protein synthesis.

Tempo Induces Chromatin Fragmentation

Chromatin fragmentation is an important hallmark of apoptotic cell death.[24,25] We performed the ultrastructural analysis of the morphologic changes induced by tempo in LNCaP cells. Figure 3 shows the features of LNCaP cells treated with tempo (5 mM, 24 hours) or ethanol (0.1%, vehicle control, 24 hours) and untreated cultures. Electron microscopy of control groups revealed distinct mitochondria, rough endoplasmic reticulum, and Golgi apparatus. In addition, heterochromatin was distributed uniformly throughout the nucleus (Fig. 3A–D). In contrast, tempo treatment resulted in aggregation and marginalization of chromatin in the nuclei of a large number of cells (Fig. 3E, F). The nuclear envelope remained essentially intact. In the cytoplasm, the Golgi apparatus and rough endoplasmic reticulum had disappeared or disrupted, and mitochondria were not discernible in a majority of tempo-treated cells (Fig. 3G). Furthermore, pro-

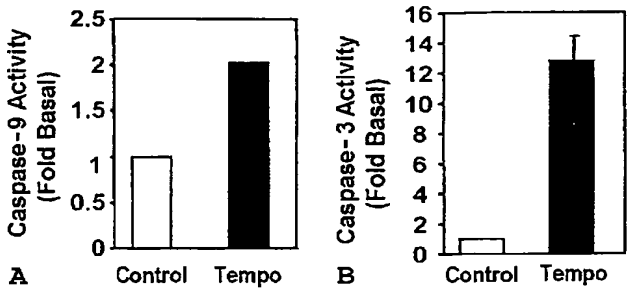

FIGURE 4. Tempo increases activities of caspase-9 and caspase-3 in LNCaP cells. LNCaP cells (3×10^6) were plated on 100-mm tissue culture dishes in 10% bovine calf serum (BCS) in RPMI overnight and then switched to 5% BCS in RPMI with or without tempo. Whole cell lysates were assayed for caspase-9 (A) and caspase-3 activities (B) as detailed in the Materials and Methods. Three independent experiments were performed. Data from representative experiments, each performed in triplicate, are shown. Control, 0.1% ethanol, 24 hours; tempo, 5 mM, 15 hours (A) and 2.5 mM, 24 hours (B).

nounced vacuolation, perhaps due to dilated endoplasmic reticulum or Golgi vesicles, was observed throughout the cytoplasm in a majority of tempo-treated cells, making it very difficult to identify membranous organelles (Fig. 3E, F). These observations provide support for an apoptotic mechanism of tempo-induced cell death.

Tempo Activates Caspase-9 and Caspase-3

We measured the activities of the caspase family of cysteine aspartate-specific proteases, caspase-9, and caspase-3 in LNCaP cells treated with tempo. An approximately 2-fold increase in caspase-9 and a 13-fold increase in caspase-3 activities were observed in tempo-treated cultures compared with control 0.1% ethanol (Fig. 4). In addition, the level of increase in caspase-3 activity appears to be cell type dependent. We found that tempo treatment (2.5 mM, 24 hours) of other human prostate adenocarcinoma cells (PC-3) led to a relatively modest activation of caspase-3 (approximately 2-fold) (data not shown). These data suggest that tempo-induced cell death may be due to activation of a caspase-dependent mechanism of apoptosis.

Tempo Enhances the Number of Cells in G_2/M Phase and Inhibits the Number of Proliferating Cells (S Phase)

Several small molecules have been shown to target key regulators of the cell cycle, promoting cell cycle arrest and apoptosis.[26–28] We examined the effect of tempo on cell cycle distribution profile in prostate carcinoma cells. A dose-dependent increase in the G_2/M population of cells was observed in all prostate carcinoma cell lines tested after treatment with tempo (0.5 mM, 24 hours: LNCaP, approximately 1.5-fold ; DU-145, approximately 1.02-fold ; PC-3, approximately 1.2-fold; 5.0 mM, 24 hours: LNCaP, approximately 5.3-fold; DU-145, approximately 1.6-fold; PC-3, approximately 1.5-fold) (Fig. 5 and Table 1). In agreement with the apoptotic effects of tempo, an increase in the sub-G_1 population of cells was observed in tempo-treated cells (Table 1). We also observed a concomitant decline in the number of proliferating cells (S phase) after treatment of cells with various doses of tempo (0.25–5.0 mM, 24 hours) (Fig. 5, Table 1, and data not shown). The inhibitory effect of tempo on the number of proliferating cells was verified independently by chemiluminescence detection of BrdU incorporation. In this assay, tempo treatment led to a dose-dependent decrease in the number of BrdU-labeled cells (Fig. 6). Consistent with the effects of tempo on cell cycle kinetics (Fig. 5 and Table 1), differences in sensitivities to tempo-induced reduction in the number of proliferating cells were observed in different cell lines (2.5 mM, 24 hours: LNCaP, approximately 5.4-fold; 2.5 mM, 48 hours: DU-145, approximately 2.4-fold; 5.0 mM, 24 hours: PC-3, approximately 2-fold) (Fig. 6). Hormone-responsive LNCaP cells were the most sensitive and PC-3 cells were least responsive to tempo-induced inhibition of cell proliferation. These data demonstrate that tempo induces G_2/M arrest and suggest that a tempo-induced decrease in the number of proliferating cells is a result of the loss of cell viability.

Therapeutic Efficacy of Tempo against Human Prostate Carcinoma

The antitumor activity of tempo was tested in athymic mice bearing LNCaP prostate tumor xenografts. Tempo treatment (intratumorally) of LNCaP tumor-bearing mice caused a significant tumor growth delay and subsequent tumor regression compared with control groups (Day 30, PBS or ethanol vs. tempo, $P < 0.05$, $n = 4$) (Fig. 7). Three of the four animals in the tempo group showed tumor regression. Regressed tumors showed no sign of reoccurrence and all animals appeared healthy during a 3-month observation period after the last tempo dosing. Tempo treatment (intratumorally) of MDA-MB 231 human breast tumor-bearing mice also caused a significant suppression of tumor growth ($P < 0.002$) (data not shown). In addition, tempo treatment (intraperitoneal via continuous infusion) of PC-3 human prostate tumor-bearing athymic mice resulted in significant tumor growth inhibition followed by tumor regression ($P < 0.007$) (data not shown).

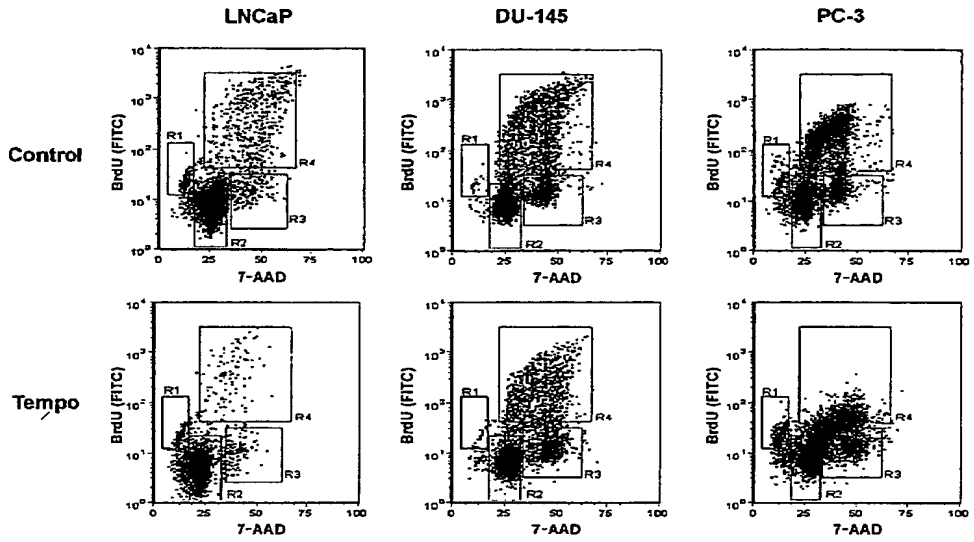

FIGURE 5. Effect of tempo on cell cycle kinetics in prostate carcinoma cells. Cells were treated with 0.5 mM tempo for 24 hours. 5'-Bromo-2'-deoxyuridine (BrdU; 10 μM) was added during the last 4 hours of tempo treatment. Control cells were left untreated. Cell cycle distribution was analyzed by flow cytometry after coupled staining with fluorescein isothiocyanate (FITC)-conjugated anti-BrdU antibody and 7-amino-actinomycin D (7-AAD) as described in the Materials and Methods. Representative histograms depict relative number of cells in the G_1 (R2), S (R4), G_2/M (R3), and sub-G_1(R1) phases of the cell cycle (LNCaP, control: G_1, 71.91%; S, 14.17%; G_2/M, 3.26%; sub-G_1, 4.55%; LNCaP, tempo: G_1, 73.68%; S, 4.91%; G_2/M, 4.87%; sub-G_1, 7.99%; DU-145, control: G_1, 72.06%; S, 12.78%; G_2/M, 12.09%; sub-G_1, 0.61%; DU-145, tempo: G_1, 62.89%; S, 19.85%; G_2/M, 12.37%; sub-G_1, 0.64%; PC-3, control: G_1, 48.09%; S, 33.48%; G_2/M, 8.55%; sub-G_1, 1.38%; PC-3, tempo: G_1, 53.69%; S, 20.79%; G_2/M, 10.24%; sub-G_1, 3.29%).

TABLE 1
Effect of Tempo on Cell Cycle Kinetics in Prostate Carcinoma Cell lines[a]

	LNCaP		DU-145		PC-3	
Cell cycle phase	Without	With	Without	With	Without	With
G_2/M	3.26%	17.24%	12.09%	19.51%	8.55%	12.95%
Sub-G_1	4.55%	8.89%	0.61%	2.7%	1.38%	7.9%
G_1	71.91%	63.86%	72.06%	70.31%	48.09%	42.73%
S	14.17%	1.51%	12.78%	3.52%	33.48%	31.26%

[a] Cells were treated with tempo (5 mM, 24 hours), and bromodeoxyuridine (10 μM) was added during the last 4 hours of tempo treatment. Cell cycle distribution profiles were determined by fluorescence-activated cell sorting as described in the Materials and Methods.

Enhanced Cytotoxic Effects of a Combination of Doxorubicin or Mitoxantrone and Tempo

We investigated the use of tempo as an anticancer agent in combination with a conventional chemotherapeutic drug, doxorubicin or mitoxantrone. Initially, in vitro dose-response and time-course experiments were performed to determine the treatment conditions at which minimum cell killing was induced with single agents (tempo or drug) (data not shown). Depending on the tumor cell type, a combination of low doses of tempo and doxorubicin caused additive or synergistic cytotoxic effect compared with single agents (percent cell death, LNCaP: tempo, 24.03 ± 12.45 %; doxorubicin, 44.83 ± 6.58%; tempo and doxorubicin, 61.27 ± 7.28%; DU-145: tempo, 7.78 ± 12.17%; doxorubicin, 34.22 ± 9.85%; tempo and doxorubicin, 55.02 ± 7.51%; PC-3: tempo, 35.84 ± 2.86%; doxorubicin, 30.92 ± 1.63%; tempo and doxorubicin, 61.56 ± 7.84%) (Fig. 8A). The combination of tempo and mitoxantrone exhibited synergistic cytotoxicity in the hormone-refractory prostate carcinoma cell lines tested (percent cell death, DU-145: tempo, 8.31 ± 4.59%; mitoxantrone, 42.57 ± 3.29%; tempo and mitoxantrone, 61.38 ± 4.86%; PC-3: tempo, 4.29 ± 7.2%; mitoxantrone, 26.32 ± 1.58%; tempo and mitoxantrone, 47.95 ± 4.60%) (Fig. 8B).

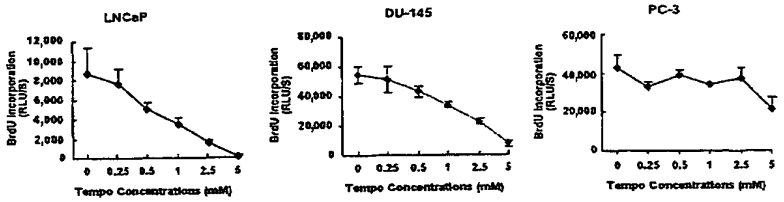

FIGURE 6. Tempo inhibits the number of proliferating prostate carcinoma cells. Cells were treated with the indicated doses of tempo for 24 hours (LNCaP and PC-3) or 48 hours (DU-145). 5'-Bromo-2'-deoxyuridine (BrdU; 10 μM) was added 2 hours before termination of tempo treatment. BrdU incorporation was measured by enzyme-linked immunosorbent assay using peroxidase-conjugated anti-BrdU antibody and chemiluminescence as described in the Materials and Methods. Control cells were treated with 0.1% ethanol. Data points represent the mean ± the standard deviation of four to six determinations per treatment group. RLU/S: relative light units/sec.

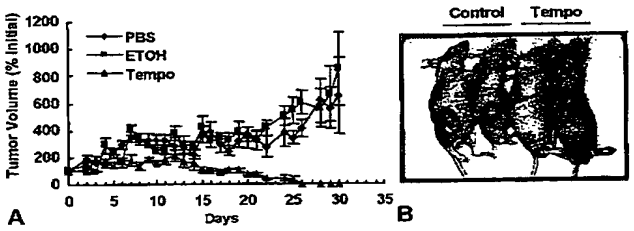

FIGURE 7. Tempo causes regression of human prostate carcinomas in athymic mice. LNCaP cells (1×10^6) were implanted (subcutaneously) in the right flank region of male athymic mice (BALB/c nu/nu) as described in the Materials and Methods. Mice with a mean tumor volume of 75–100 mm^3 were randomized to 3 treatment groups per tumor model. (A) LNCaP tumor-bearing mice received tempo (intratumorally) daily (100 mg/kg: × 8, Days 1–8; 200 mg/kg: × 15, Days 9–23). Control groups received either phosphate-buffered saline (PBS) or 4% ethanol in PBS on the same dosing schedule as tempo. (B) Representative LNCaP tumor-bearing control mice (left two) and tempo-treated mice (right two) on Day 28. Experiment was terminated as scheduled. Each data point represents the mean ± the standard error ($n = 4$). The experiment was repeated twice with comparable results.

DISCUSSION

Design and development of membrane-permeable, small molecules targeting cell death or cell survival signaling pathways have profound clinical implications. In the current report, we demonstrate apoptotic effects of tempo, a low molecular weight membrane-permeable nitroxide, in hormone-sensitive and hormone-refractory prostate carcinoma cells. Hormone-responsive LNCaP cells were significantly more sensitive to tempo-induced apoptosis compared with the hormone-resistant cell lines tested (PC-3 and DU-145) (Fig. 1). Electron microscopy of LNCaP cells exposed to tempo revealed pronounced mitochondrial damage and chromatin condensation (Fig. 3). EPR studies indicate that different tumor cell lines may have different rates of tempo signal decay.[29] Differences in the sensitivity of different tumor cell lines to tempo observed may, in part, depend on the rate of tempo decay with slower decay signals associated with a higher sensitivity.

Tempo-induced cell death appears to require macromolecular synthesis because CHX pretreatment led to a partial or complete restoration of cell viability in tempo-treated cancer cells (Fig. 2). Such antimetabolites may target one or more components of cell death pathway. For example, tempo treatment leads to increased levels of ceramide.[19] Ceramide-induced apoptosis has been associated with activation of caspase-9 and caspase-3,[30] and CHX has been shown to inhibit ceramide-induced apoptosis in prostate carcinoma cells.[31] In addition, nitroxide compounds including tempo have been associated with increased oxidative stress including depletion of intracellular glutathione pools and an increase in intracellular concentrations of $O_2^{\cdot}$ and hydrogen peroxide (H_2O_2).[15,29] Treatment of human hepatoma cells with either ACT-D or CHX was shown to block H_2O_2-induced apoptosis.[32]

Activation of caspase-9 and caspase-3 has been recognized as hallmarks of mitochondrial cell death in a variety of different cell types.[33] In agreement with differences in the levels of apoptosis observed in hormone-responsive and unresponsive prostate carcinoma cells, tempo treatment induced a relatively higher caspase-3 activity in LNCaP cells compared with PC-3 cells (Fig. 4, and data not shown). Tempo treatment increases ceramide levels,[19] and ceramide enhances oxidative damage by inducing caspase-3–dependent proteolysis of catalase and enhancing accumulation of reactive oxygen species.[34] Gariboldi et al.[35] reported that expression of p21 WAF/CIP1, a cell cycle arrest-related protein induced by p53, was associated with nitroxide free radical tempol (4-hydroxy-2, 2, 6, 6-tetramethylpiperidine-1-oxyl)-induced apoptosis in C6 glioma cells. LNCaP cells have wild type p53, whereas PC-3 and DU-145 cells have mutated p53,[36] suggesting that tempo may induce a p53-de-

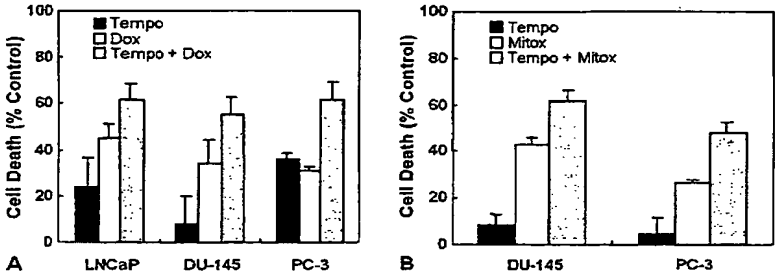

FIGURE 8. Enhanced in vitro efficacy of a combination of tempo and doxorubicin or mitoxantrone. (A) Enhanced cytotoxic effects of a combination of tempo and doxorubicin. Tumor cells were treated with tempo (LNCaP, 0.5 mM, 24 hours; DU-145, 1 mM, 24 hours; PC-3, 1 mM, 48 hours) in the presence or absence of doxorubicin (LNCaP, 0.5 μg/mL, 24 hours; DU-145, 100 ng/mL, 24 hours; PC-3, 1 μg/mL, 48 hours). Ethanol (0.1%) served as the vehicle control. Cell death was measured by WST-1 assay as described in the Materials and Methods. Values shown are the mean ± the standard deviation (SD) of six determinations. Data are a representative of two to three independent experiments. (B) Enhanced cytotoxic effects of a combination of tempo and mitoxantrone. Cells were treated with tempo (DU-145, 1 mM, 24 hours; PC-3, 0.5 mM, 48 hours) in the presence or absence of mitoxantrone (DU-145, 100 ng/mL, 24 hours; PC-3, 100 ng/mL, 48 hours). Ethanol (0.1%) served as the vehicle control. Cell death was measured by WST-1 assay as described in the Materials and Methods. Values shown are the mean ± SD of six determinations in a representative of two to three independent experiments.

pendent and/or p53-independent mechanism of cell death. Our data suggest that tempo induces G_2/M arrest, loss of cell viability, and a decrease in the number of proliferating cells (Figs. 2, 5, 6, and Table 1). It appears that tempo may induce a multifactorial cell death response, including enhanced oxidative damage after ceramide generation and activation of potent cell death effectors such as caspase-3, and cell cycle arrest. In addition, as yet unknown effectors may play a role in tempo-induced apoptosis. Accordingly, tempo treatment of prostate and breast carcinoma cells leads to a differential expression of several known and unknown genes, some of which are known to play a role in stress response and energy metabolism.[38] Furthermore, tempo treatment of prostate and breast cancer cells leads to down regulation of p55/CDC20 and CDH1 proteins, known activators of anaphase promoting complex (APC).[39]

We demonstrate that tempo possesses significant antitumor activity against representative hormone-responsive (LNCaP) and hormone-refractory prostate tumor xenografts grown in athymic mice (PC-3) (Fig. 8, and data not shown). Tumor-bearing mice treated with tempo maintained normal body weight and appeared to be clinically healthy. Systemic therapy using a combination of a chemotherapeutic drug and an agent that activates a cell death-specific signal(s) may improve the clinical outcome in patients with metastatic prostate carcinoma.[2] We determined the effect of a combination of tempo and doxorubicin or mitoxantrone on cell viability in a representative prostate tumor cell line. In vitro data show significantly enhanced cytotoxic effects of both drugs in combination with tempo, albeit in a cell type-specific manner (Fig. 8).

In conclusion, the current study demonstrates that nitroxide tempo is a novel compound with significant apoptotic and antitumor activities against hormone-dependent and hormone-independent prostate tumor models. Tempo also acts as a chemosensitizer in vitro, and in vivo (data not shown). Tempo has a very short half-life,[16, 38] and relatively higher concentrations of the drug may be needed to achieve a therapeutic dose. Future investigations are necessary to elucidate the effectors of the tempo-induced cell death pathway and to further develop a prostate-targeted and clinically viable formulation of this small molecule.

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